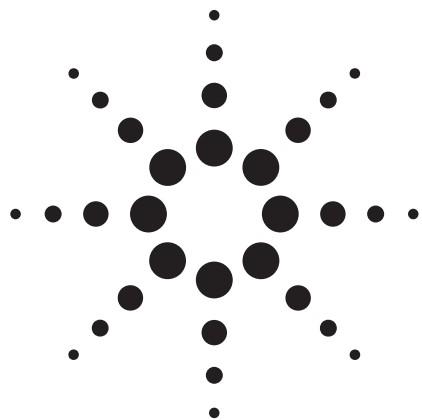


# The Use of Accurate Mass, Isotope Ratios, and MS/MS for the PPCPs in Water



## Application

### Environmental

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## Abstract

**An Agilent 6510 Quadrupole Time-of-Flight Mass Spectrometer (QTOF) is used to analyze several surface water samples for the presence of pharmaceutical compounds. A simple gradient elution is carried out on a Rapid Resolution High Throughput Extend C18 column (particle size 1.8  $\mu\text{m}$ ). Of 54 potential compounds, as many as 11 are identified in one of the samples using an algorithm known as the Agilent Molecular Feature Extractor (MFE). To make comparisons among several samples, another algorithm, known as Mass Profiler, is applied to the data processed by the MFE. Since the MFE may generate thousands of potential compounds known as features, Mass Profiler makes statistical comparisons of the features between two different samples to determine what is unique and what is common. All of this work is done with the full-scan mass spectral data. When compounds of interest are determined, accurate mass full-scan MS/MS**

**can be invoked for structural elucidation. The results of full-scan MS/MS applied to caffeine are included as an example and are relevant because many medications include caffeine as an ingredient.**

## Introduction

During the three decades prior to the year 2000, the study of chemical pollution was confined primarily to pesticides. Following a seminal article by C. Daughton [1], this focus began to shift to the emerging environmental concern for pharmaceuticals and personal care products (PPCPs). Many of these pharmaceuticals, including estrogen, have been known as endocrine disruptors, or chemicals that disrupt the physiological function of hormones in organisms. In 2004 a report from the United States Geological Survey [2] was made as a result of discovering a high preponderance of intersex (male fish exhibiting female characteristics) in smallmouth bass of the Potomac River.

The USGS has found pesticides, flame retardants, and personal-care products containing known or suspected endocrine-disrupting compounds in the Potomac River. Many of these compounds continue to be known as emerging contaminants because they are still being discovered and don't exist on any currently regulated target lists. As such, it is important to use adequate techniques to help identify these compounds and possible metabolites.

Using accurate mass in full-scan (mass range) mass spectrometry (MS), compound empirical formulas can be determined for purposes of identification. Furthermore, the high degree of spectral resolution allows for selective identification among co-eluting compounds. Isotope ratios are an additional tool because they help identify com-



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pounds with high carbon numbers as well as those that contain elements like chlorine and sulfur. Although these tools do a lot to confirm chemical formula, it may still be left to the user to decide which of the possible structures of isobaric compounds apply.

To assist in the analytical need for structural elucidation, selective MS/MS by using the quadrupole time-of-flight mass spectrometer (QTOF) is implemented. Because the Agilent QTOF also has very accurate mass at the MS/MS level, it is easier to determine the structures of the product ions, which correspond as substructures of the precursor ion and thereby reduce the number of possible structures pertaining to the derived empirical formulas from several to one.

The list of pharmaceuticals to look for in the environment is ever-increasing and many of them are metabolites with unknown structures. Identifying these compounds requires the technology of the QTOF. Furthermore, the fast scanning capability is necessary for identifying 10s to 100s of these compounds in samples with relatively short run times. The Agilent QTOF is capable of acquiring full-scan MS data at the rate of 20 spectra/sec. The resulting large amount of data representing a possibly large number of compounds needs to be converted into useful information. The Agilent Molecular Feature Extractor (MFE), which is a standard part of the MassHunter Qualitative Analysis software, carries out the following steps:

- Persistent chemical background removed

- Co-eluting interferences resolved
- Isotopic clusters recognized and grouped
- 2D/3D data visualization
- Chemical identification (accurate mass, isotope matching)
- Database searching (NIST, ChemIDPlus)

In addition to applying the algorithm Mass Feature Extractor to pull out the features from the chromatographic data, which could be compounds, another algorithm, known as Mass Profiler, is applied to the list of features among different samples to determine differences and commonalities. Each sample is injected three times, or multiple samples from the same source could be used to determine what is statistically consistent in terms of the features derived for the sample by MFE. The result is called a group. Two groups representing two different sample sources can then be compared to see what features differ, are unique, or are common, and, if common, whether they differ in abundance.

A batch of water samples is filtered and extracted using solid-phase extraction, which resulted in an approximate 1,000-fold increase in concentration. Samples analyzed in this work are believed to contain compounds at the 10 to 100 ppb level, which corresponds to the 10 to 100 ppt range in the original water sample. The compounds that may be in these samples are included with their exact neutral masses in Table 1.

**Table 1. List of Compounds with Corresponding Neutral Masses That May Be in a Given Sample**

Compound	Neut. mass	Compound	Neut. mass	Compound	Neut. mass
Acetaminophen	151.06333	Diphenhydramine	255.16231	Paroxetine	329.14272
Albuterol	239.15214	Duloxetine	297.11873	Ranitidine	314.14126
Aspirin	180.04226	Enalaprilat	348.16852	Sertraline	305.07380
Bupropion	239.10769	Erythromycin	573.51210	Simvastatin	418.27192
Caffeine	194.08038	Fluoxetine	309.13405	Sulfachloropyridazine	284.01347
Carbamazepine	236.09496	Fluvoxamine	318.15551	Sulfadimethoxine	310.07358
Cimetidine	252.11572	Furosemide	330.00772	Sulfamethazine	278.08375
Clofibric acid	214.03967	Gemifrozil	250.15698	Sulfamethizole	270.02452
Citalopram	324.16379	HCTZ	296.96447	Sulfamethoxazole	253.05211
Codeine	299.15215	Ketoprofen	254.09429	Thiabendazole	201.03607
Cotinine	176.09496	Miconazole	413.98602	Triclocarban	313.97805
Dehydronifedipine	344.10084	Naproxen	230.09429	Triclosan	287.95116
Diclofenac	295.01668	Norfluoxetine	295.11840	Trimethoprim	274.14298
Diltiazem	414.16133	Norsertaline	293.05000	Venlafaxine	267.12593
		1,7-dimethylxanthine	180.06473	Warfarin	308.10486

## Experimental

### Sample Preparation

Prepared samples are provided by the United States Geological Service National Water Quality Laboratory (USGS/NWQL) in Denver, Colorado. The details of the procedure used are not included in this application, but are available upon request. Pharmaceuticals are typically extracted from surface water by using disposable polypropylene syringe cartridges that contain 0.5 g of polymeric sorbent. One liter of sample is pumped through the solid-phase extraction (SPE) cartridge. The analyte material is later eluted into 1 mL of methanol, resulting in a concentration increase of three orders of magnitude.

### LC/MS Method Details

#### LC Conditions

Agilent 1100 Series binary pump, degasser, wellplate sampler, and thermostatted column compartment

Column	Agilent ZORBAX RRHT Extend C18 2.1 mm × 50 mm, 1.8 μm Agilent p/n: 727700-902		
Column temperature	40 °C		
Mobile phases	A = 0.1% formic acid in water B = 0.1% formic acid in acetonitrile		
Flow rate	0.3 mL/min		
Injection volume	5 μL		
Gradient			
	Time (min)	%B	
	0.0	0	
	10.0	67	Stop time: 15 min
	11.0	100	Post run: 10 min

#### MS Conditions

Mode	Positive electrospray ionization using the Agilent G3251A Dual ESI source
Nebulizer pressure	40 psig
Drying gas flow	9 L/min
Drying gas temp.	350 °C
V <sub>cap</sub>	3500 V
Scan range	<i>m/z</i> 50–1000
Scan speed	1 scan/sec

#### MS/MS Conditions

Collision energy	30 V
Scan range	<i>m/z</i> 50–1000
Scan speed	1 scan/sec

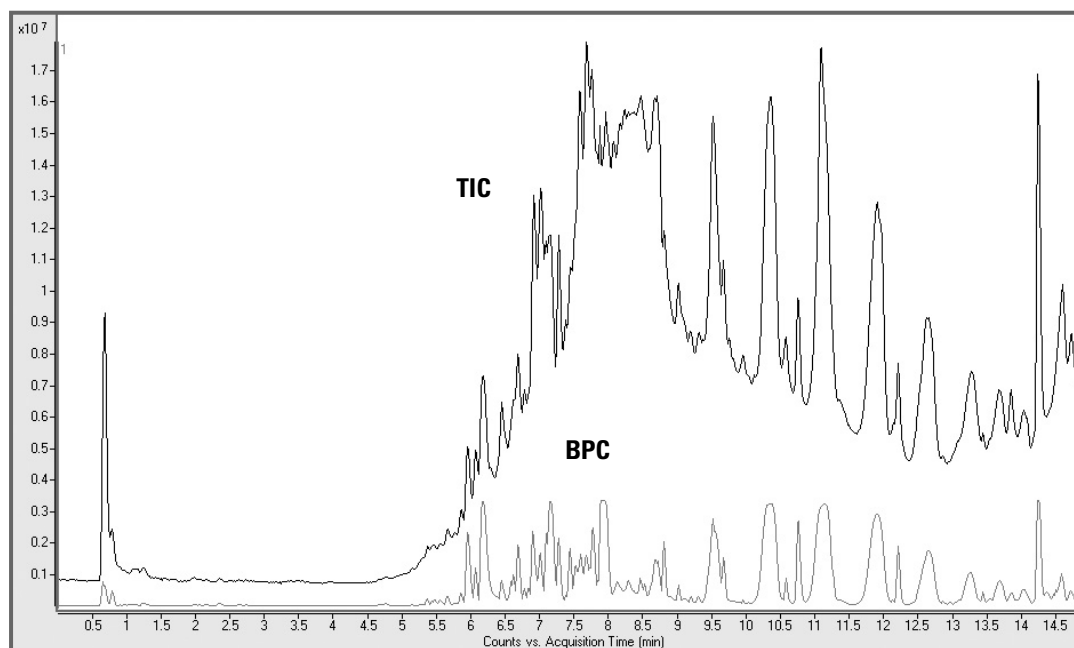
## Results and Discussion

Of the several samples analyzed, results for Samples 4 and 10 will be considered here. To get an idea of the task at hand, an overlay of the total ion and base peak chromatograms for the first injection of Sample 4 is shown in Figure 1. The base peak chromatogram is generated to help the analyst identify peaks in the chromatogram corresponding to real compounds. Figure 2 shows the spectrum at the apex of one such peak. Note the complexity of this spectrum and the difficulty involved in not only determining which spectral peaks are of value, as they may pertain to co-eluting compounds, but then having to apply this reasoning to several peaks in the chromatogram.

Applying the algorithm of the Molecular Feature Extractor program to this data file results in the display of the processed chromatogram and the corresponding contour plot shown in Figure 3. The upper left-hand chromatogram is the unprocessed TIC, same as shown in Figure 1. On the right is the processed chromatogram after applying the steps listed in the Introduction. Random background noise has been removed. Below each of these chromatograms are shown the corresponding contour plots, which are the presentations of spectral data points in an *m/z* versus chromatographic retention time plots. The contour plot at the lower left-hand corner of the display shows a very dense distribution of data points, most of which correspond to random noise.

The contour plot at the lower right-hand corner is the result of processing the data so that a significant amount of molecular features are derived for closer examination. In fact, using the following settings for filtering the data, some 5,431 features are derived for this sample in the first injection:

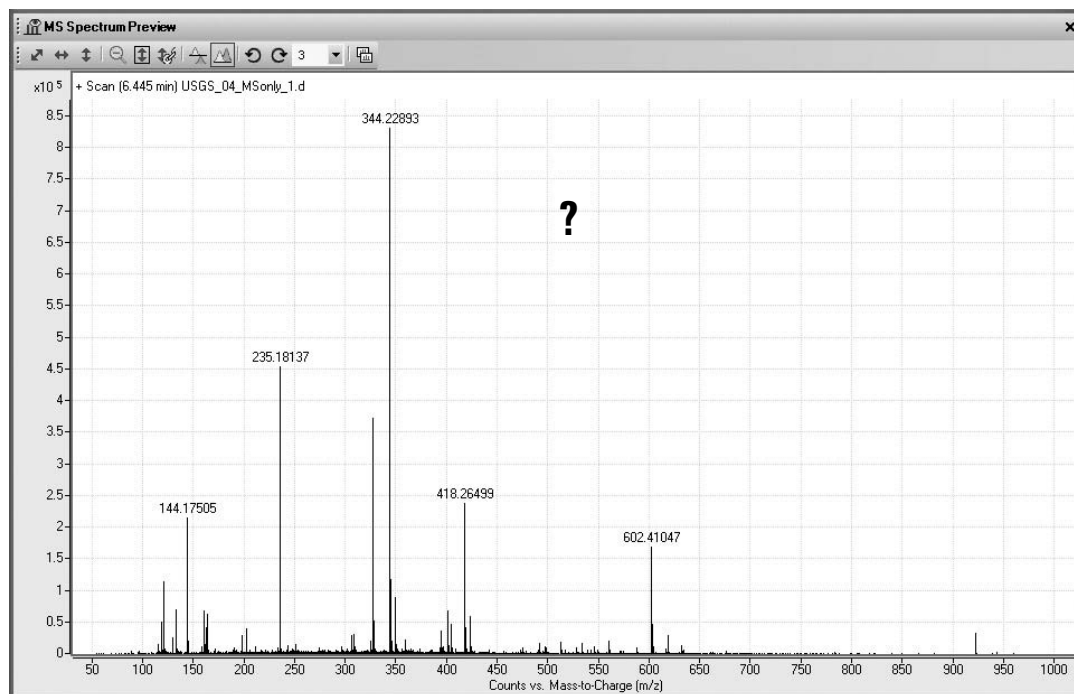
- Spectral S/N > 2
- Mass range: *m/z* 150 to 800
- [M + Na]<sup>+</sup> and [M + NH<sub>4</sub>]<sup>+</sup> adducts considered
- Relative intensity in the spectrum > 0.1%
- Each feature must contain at least 2 ions



**Figure 1. Overlay of total ion chromatogram (TIC) and base peak chromatogram (BPC) for Sample 4.**

If we now investigate some of the features that have been found we can begin with the peak apex spectrum examined in Figure 2. The retention time is 6.445 minutes and MFE has derived features at 6.448 minutes as shown in Figure 4. The unprocessed spectrum at the top of the figure matches

that of Figure 2. However, removing random noise and using the filtering rules above a processed spectrum containing 12 features is derived and shown at the bottom. A subset of the list of features is shown at right.



**Figure 2. Spectrum at apex of base peak with retention time of 6.445 minute.**

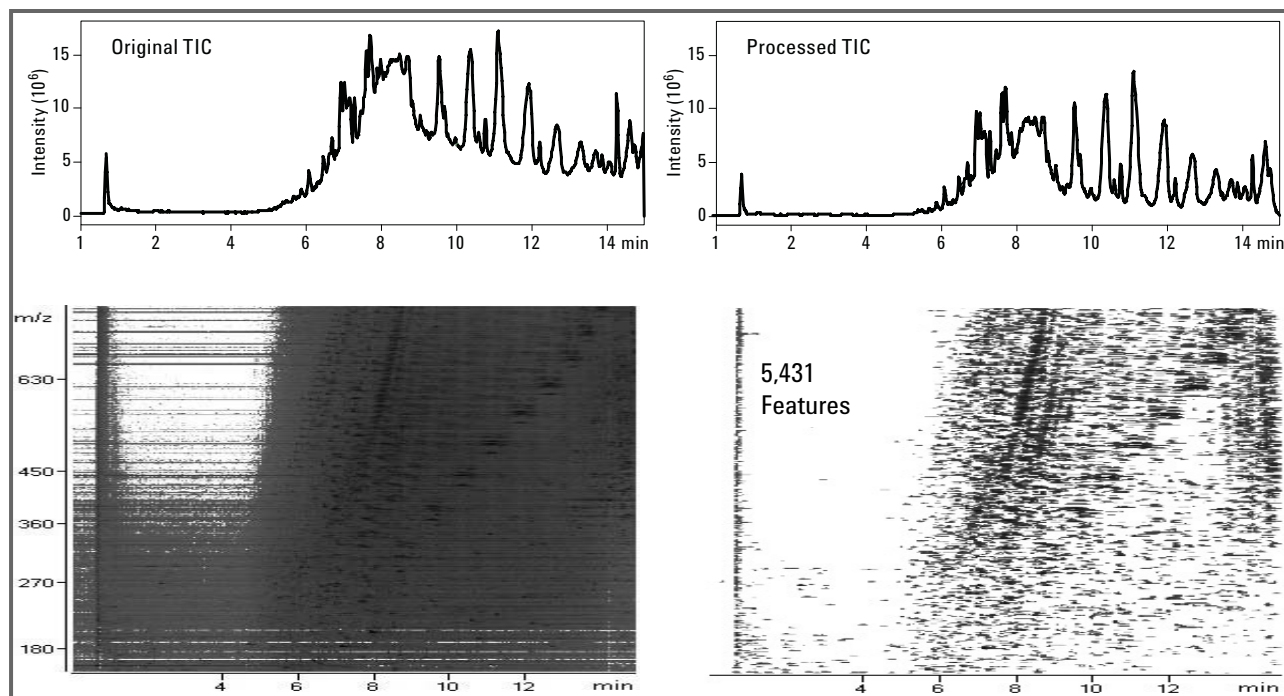


Figure 3. Both unprocessed and processed data of Sample 4 using MFE.

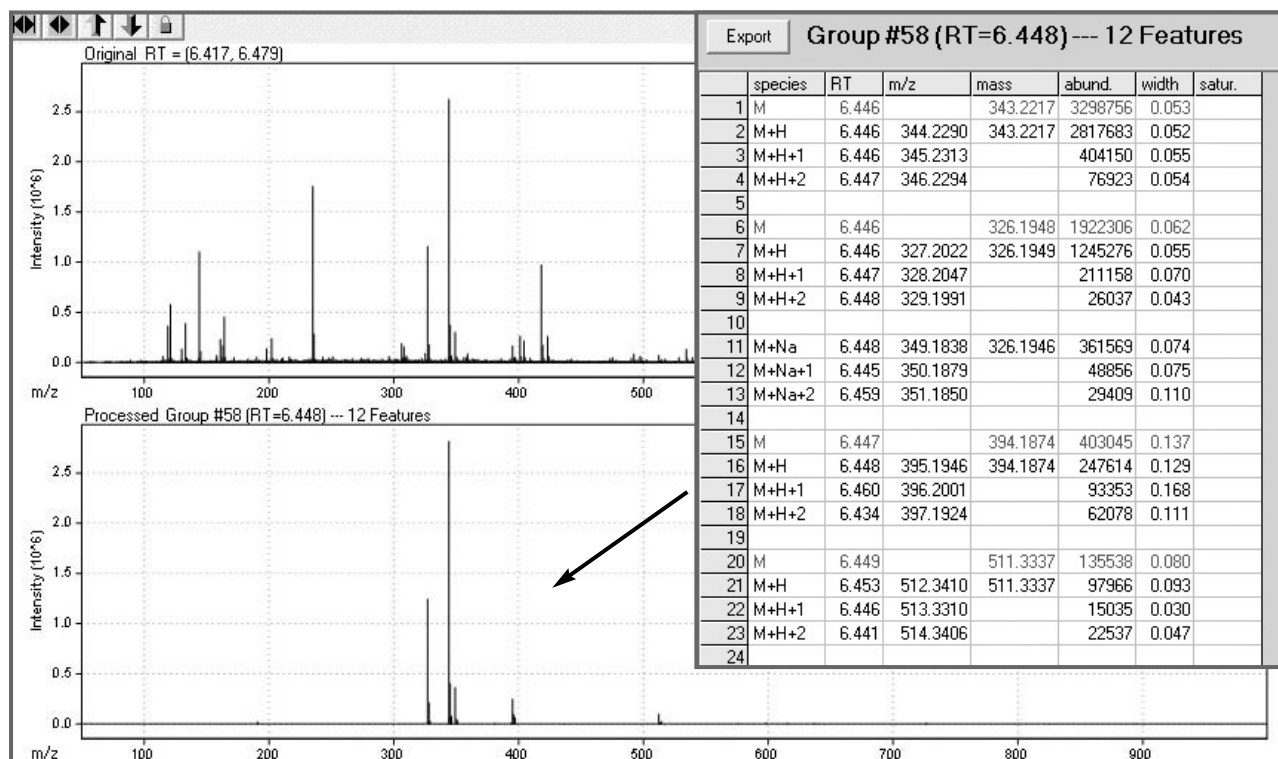


Figure 4. Twelve features shown at derived retention time of 6.448 minutes.

If we want to filter the data to only show compounds corresponding to the list at the beginning of this article, we can place the neutral masses into an inclusion list of MFE as shown in Figure 5. We also assume that the compounds of interest do not elute until after 4 minutes and the mass range of interest is 150 to 600, which corresponds to the compounds of Table 1.

After applying the filtering of data with the compound list shown in Figure 5, eight features appear to be found in Sample 4 as shown in Figure 6. The corresponding chromatogram containing these eight features is also shown.

Before looking more closely at any one of these compounds, the data of Sample 4 is now going to be compared with data from another sample, Sample 10. The comparison will be carried out using an algorithm known as Mass Profiler. In

order to use Mass Profiler, at least three injections of each sample must be made to determine what is consistently there and what is random and should be disregarded. In this work each sample is injected three times. The data is first processed by MFE to generate features. Mass Profiler filters out features that are inconsistent among the three injections for each sample. The resulting data is called a Group. Therefore, in comparing Samples 4 and 10 Mass Profiler will be referring to them as Group 4 and Group 10.

In Figure 7, Mass Profiler shows a plot of features common to both Groups 4 and 10 and displays them as mass versus retention time. By clicking on any one of the feature points in the display, one can see the common feature for both Groups along with possible empirical formulas for the derived neutral mass.

**Display Filters**

**Feature Position**

☐ Use all the available data

Min Max

RT 4.0 15.0 min.

Mass 150 600 Da

**Special Masses**

☐ Exclude ☒ Limit to these Tolerance 0.0050 Da

284.0135	287.9512	293.0500	295.0167	295.1184
296.9645	297.1187	299.1521	305.0738	308.1049
309.1341	310.0736	313.9781	314.1413	318.1555
324.1638	329.1427	330.0077	344.1008	348.1685
413.9860	414.1613	418.2719	573.5121	

Masses from Table 1

**Isotope Pattern**

Formula Custom

formula

normalized height error 0.15

**Mass Defect**

☐ Peptide Like

target defect = 0.00 (Da) + 0.00000 x mass

deviation allowed 0.50 Da

**Features of Unknown Mass**

☒ included ☐ excluded ☐ limited to these

**Number of Ions**

☒ >= 2 ☐ <

**Abundance**

Relative Absolute Top

Min Relative Abundance 0.100 %

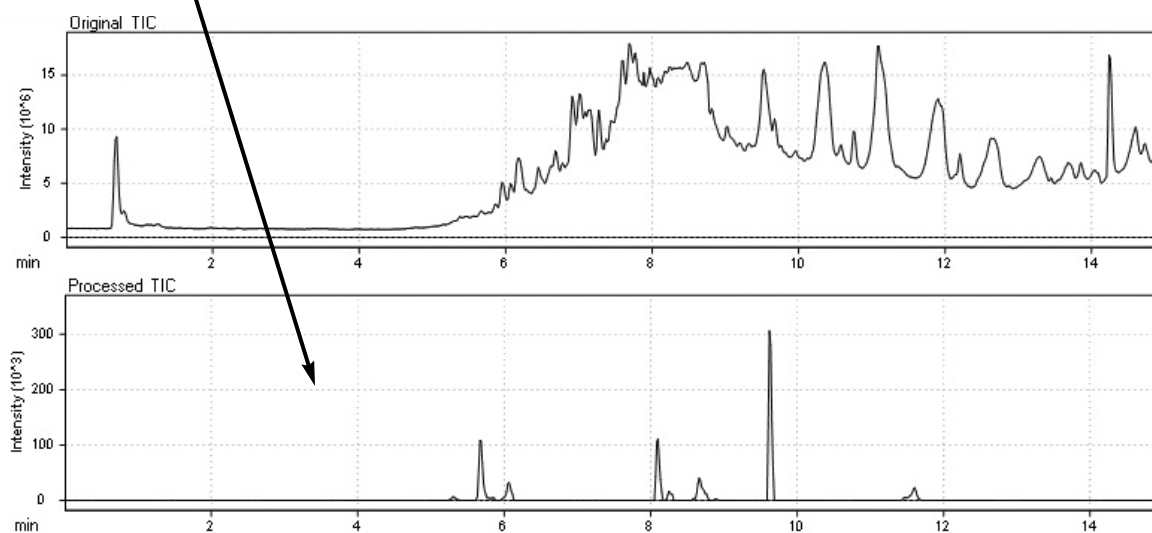
**Neutral Losses**

Tolerance 0.0050 Da

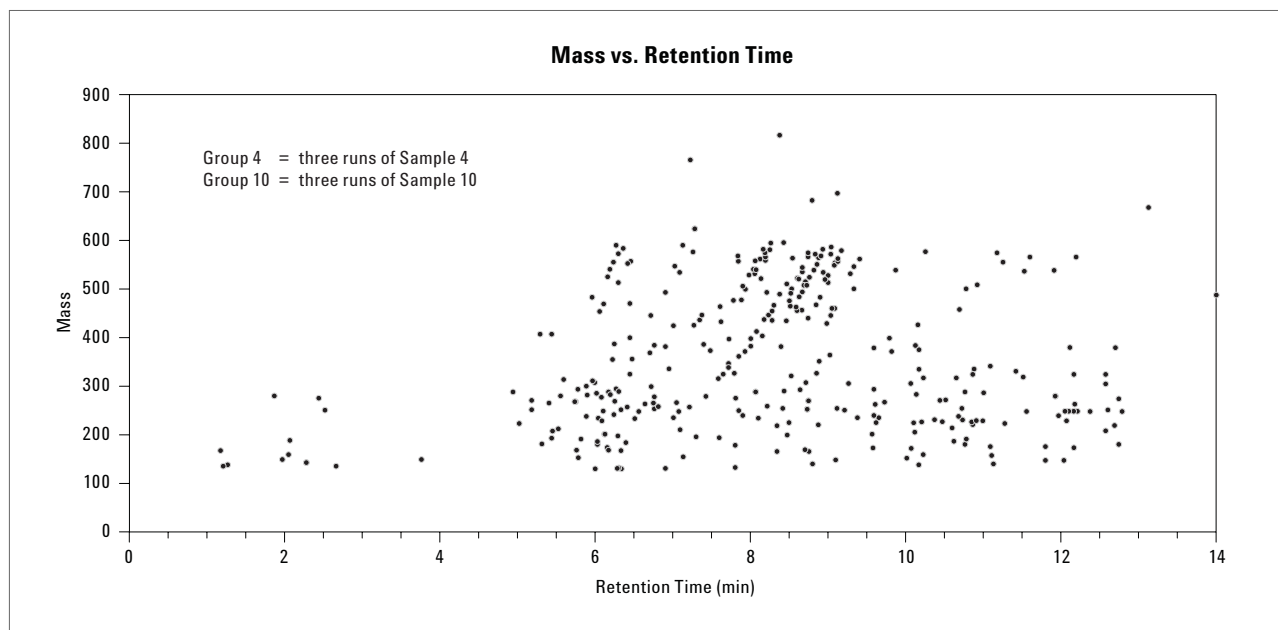
OK Cancel

**Figure 5.** Display filter settings for finding features that match compound list of Table 1.

Export features:8 / groups:8													
	Group	RT	mass	mass SD	abund.	satur.	height	#ions	minZ	maxZ	#z	width	#features
1	1	9.629	236.0961		915731		267469	2	1	1	1	0.045	1
2	2	5.678	299.1536		431858		91085	2	1	1	1	0.083	1
3	3	8.095	236.0961		382835		96590	2	1	1	1	0.052	1
4	4	8.671	299.1527		229538		23883	3	1	1	1	0.121	1
5	5	6.064	299.1525		146722		26146	2	1	1	1	0.076	1
6	6	11.598	250.1607		134252		19366	2	1	1	1	0.128	1
7	7	8.262	252.1204		63528		12743	2	1	1	1	0.058	1
8	8	5.307	296.9660	0.0019	28147		3522	4	1	1	1	0.072	1



**Figure 6.** Eight features found corresponding to the neutral masses of Table 1. Corresponding processed chromatogram also shown.



**Figure 7.** Features present in both Groups 4 and 10 total 346.

For example, in comparing features between the two sample groups a differential analysis plot can be generated as shown in Figure 8. In this plot, the features of Group 10 that are more or less abundant than the corresponding features in Group 4 are represented. More specifically, at a retention time of 8.495 minutes there is a data point in Figure 8 that corresponds to a feature in Group 10 that is approximately 4× intensity over the corresponding feature in Group 4, which corresponds to

a log 2 ratio of 2. By clicking on this data point in the display of Figure 8 one can see that this feature is identified as diphenhydramine, with a chemical formula of  $C_{17}H_{21}NO$  and accurate mass of 0.7 ppm. See Figure 9.

With Mass Profiler it is also possible to compare two samples in terms of what features are in one sample that are not in the other. In Figure 10 we see such a comparison.

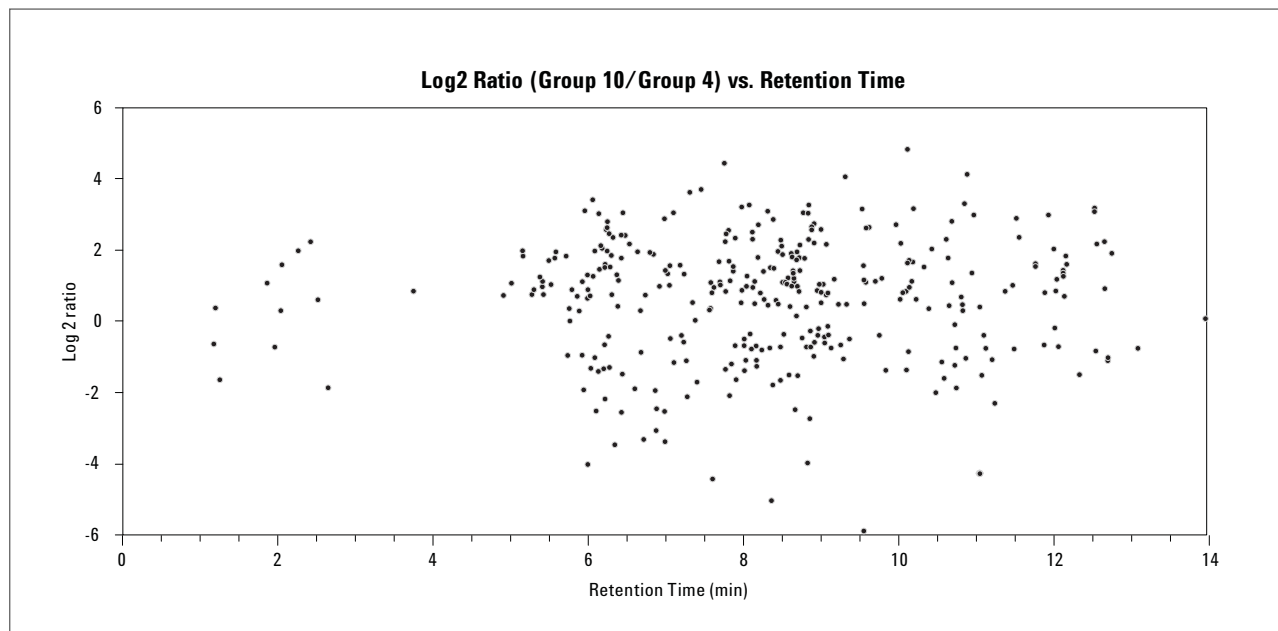


Figure 8. Features common to Groups 4 and 10 but differing in magnitude.

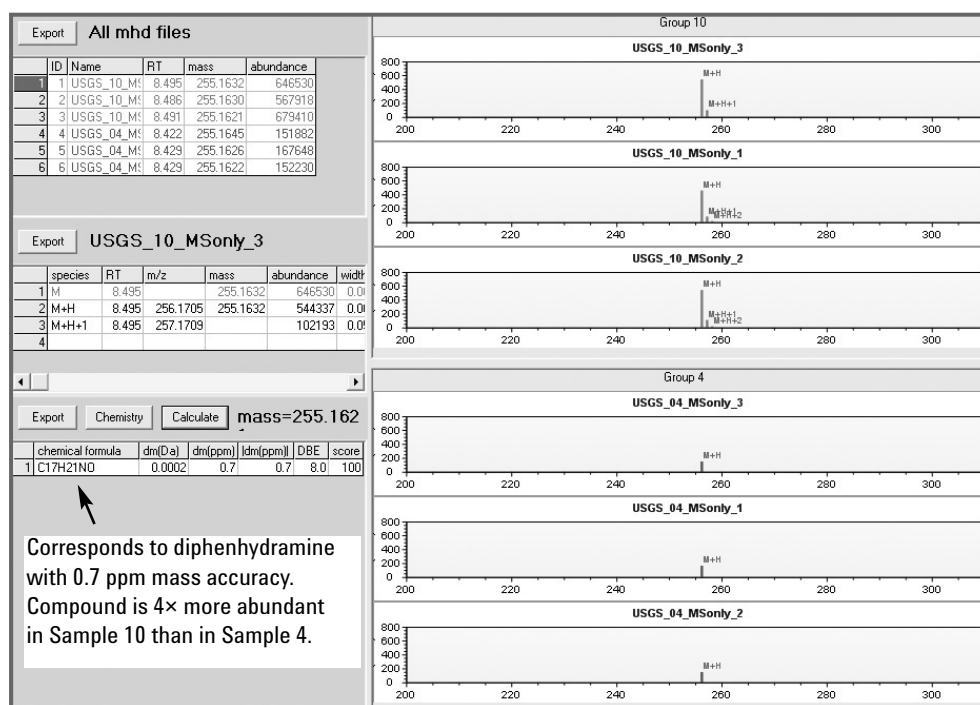
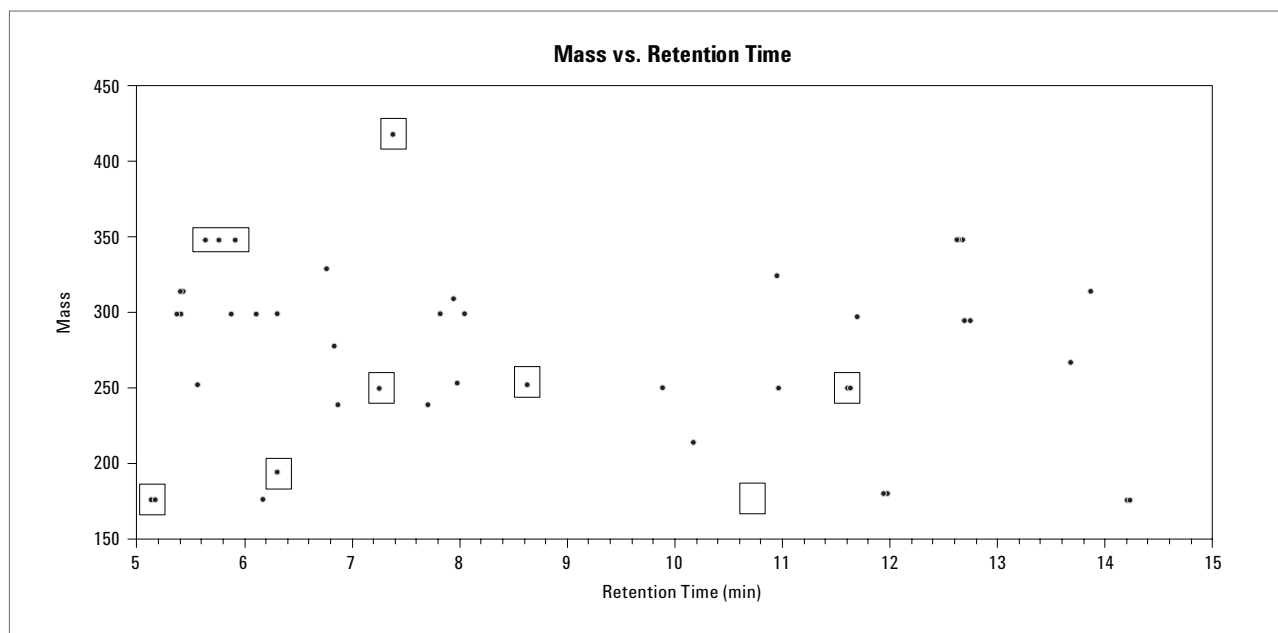


Figure 9. Feature at 8.495 minutes retention time corresponds to diphenhydramine.



**Figure 10. Features only in Group 4 (highlighted with boxes) or in Group 10.**

Mass Profiler has determined that there are 33 features only in Group 4 or in Group 10 and are not common to the two samples. Since the display in Mass Profiler is in color the features exclusive to Group 4 are blue and the features exclusive to Group 10 are red. Since Agilent applications are normally published in black and white, boxes have been placed around the blue features for Group 4 for viewing convenience.

So far, all of the data have been collected in full-scan MS mode. Once features are identified as compounds needing more structural information, or it is of interest to perform some quantitation, a targeted MS/MS analysis can be performed in which the ion mass of the feature is considered as precursor ion and fragmented to form accurate mass product ions. The accurate mass of these product ions can determine their chemical formula and possible structures. Because the QTOF also has a high degree of spectral resolution in MS/MS mode, very narrow extracted ion chromatograms may be generated for each ion and then summed together for quantitation signal.

In Figure 11 we see the accurate mass MS/MS fragmentation of caffeine using the MS/MS settings noted in the LC/MS Method Details. Caffeine is of environmental interest because many medications contain it as an ingredient. Chemical formulas for each product ion is derived based on the possible arrangements of C, H, N, and O. Knowing the structure of caffeine, structures of the fragment ions can be proposed using their corresponding chemi-

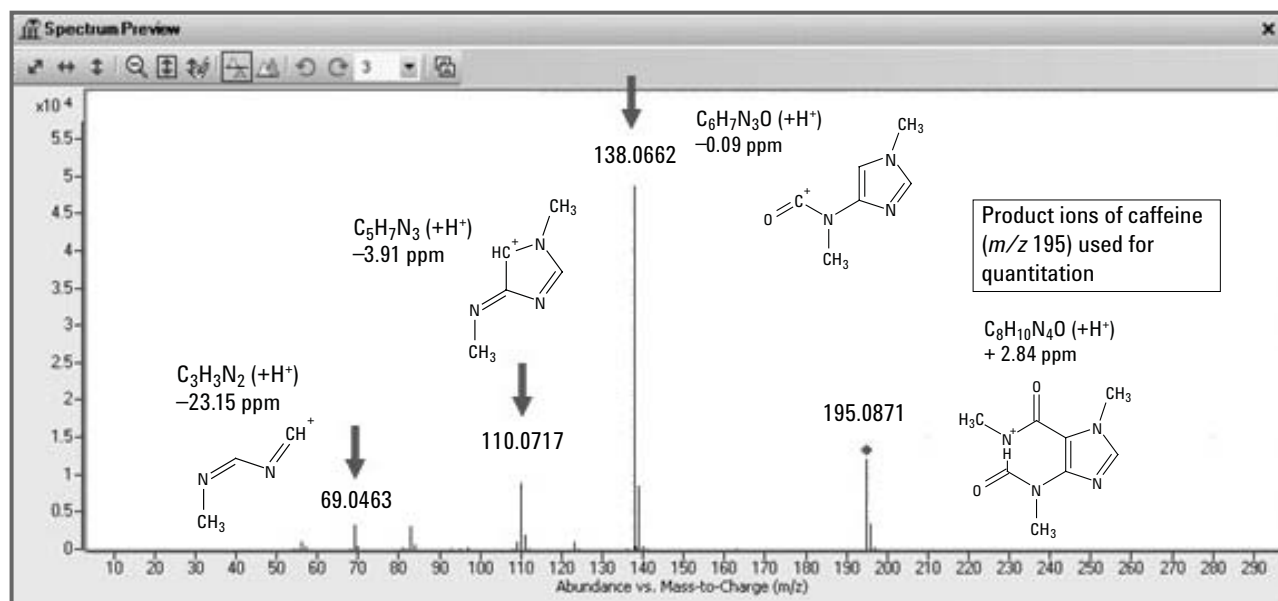
cal formula. The fragment structures are generated using ACD/MS Fragmentor (ACD Labs Release v. 10, Advanced Chemistry Development, Inc., Toronto, ON, Canada).

## Conclusions

The QTOF is an excellent instrument for identifying compounds using accurate mass in full-scan MS and MS/MS. Accurate mass leads to chemical formula, which can also give structural information when forming product ions in MS/MS. As a lot of data is acquired by this type of instrument to look at samples that may contain large amounts of known and unknown compounds, it is important to have algorithms like Molecular Feature Extractor that can filter usable features out of the chemical background. These features are generated from spectra as a result of removing random background signal and finding clusters of isotopes that make sense.

While this analysis is useful for one sample it may also be important to make comparisons among multiple samples as well. Another algorithm known as Mass Profiler makes such comparisons. More specifically, comparisons such as what is common to two samples and how they differ in amount. Or, what features are in one sample that aren't in the other. Once the feature is considered for more investigation, targeted MS/MS may be carried out on that feature to get structural information based on the generation of product ions.

Calculate chemical formula given accurate mass measurement and using elements C, H, N, and O



Proposed Structures

Figure 11. Targeted MS/MS mode for caffeine-producing product ions that may be used for structural elucidation as well as quantitation.

## References

1. C. G. Daughton and T. A. Ternes, "Pharmaceuticals and Personal Care Products in the Environment: Agents of Subtle Change?," *Environmental Health Perspectives*, 107, Suppl. 6, Dec 1999.
2. D. B. Chambers and T. J. Leiker, "A Reconnaissance for Emerging Contaminants in the South Branch Potomac River, Cacapon River, and Williams River Basins, West Virginia, April–October 2004," Open File Report 2006-1393, United States Geological Survey, <http://pubs.usgs.gov/of/2006/1393/>.

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